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Malondialdehyde Binding to Proteins Dramatically Alters Fibroblast Functions

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The regulation of cell metabolism by the surrounding environment is deeply altered by the posttranslational nonenzymatic modifications of extracellular proteins that occur throughout lifespan in vivo and modify their structural and functional properties. Among them are protein adducts formed by components generated from oxidative processes, such as malondialdehyde (MDA). We have investigated here the effects of MDA-binding to proteins on cultured fibroblast functions. Type I collagen and/or serum proteins were incubated with 0–100 mM MDA for 3 h before use in fibroblast cultures. In tridimensional lattice cultures, MDA-treated collagen inhibited the contracting activity of fibroblasts. A similar inhibition of lattice contraction was reproduced by the addition of MDA-treated serum to the culture medium. In monolayer cultures, the addition of MDA-modified serum proteins completely inhibited fibroblast multiplication without effect on initial adhesion steps. MDA-modified proteins decreased the proliferative capacities of cells, strongly altered cell cycle progression by blocking passage to G_2/M phases, and induced apoptotic features in fibroblasts. Our results show, for the first time, that MDA-modified proteins are potentially as deleterious as free MDA, and could be involved in aging as well as in degenerative complications of diseases with increased oxidative stress such as diabetes mellitus or atherosclerosis. J. Cell. Physiol. 191: 227–236, 2002. © 2002 Wiley-Liss, Inc.

It is well established that cell functions are controlled by complex interactions with extracellular components that trigger various transduction pathways (Juliano and Haskill, 1993; Langholz et al., 1997; Lee et al., 2000). Both soluble components, such as growth factors or cytokines, and extracellular matrix macromolecules are involved in these processes (Nathan and Sporn, 1991; Raghow, 1994; Sage, 2001). Many posttranslational modifications occur throughout protein lifespan in vivo, mainly represented by generation of adducts at specific reactive sites, the most common reaction being the nonenzymatic glycation, characterized by the binding of sugar moieties to amino groups of proteins (Reiser, 1998; Bailey and Paul, 1999). Glycation-derived adducts have been recognized for about two decades as potent agents of protein modifications, altering their structural and functional properties, especially in the case of long-lived proteins such as collagens (Bai et al., 1992; Tian et al., 1996). This nonenzymatic reaction is increased in diabetes mellitus (Vlassara, 1997), inducing significant alterations of protein–protein and cell–protein interactions (Haitoglou et al., 1992; Bobbink et al., 1997; Rittié et al., 1999). Glycation involves oxidative steps (Jiang et al., 1990), and most of the glycation end products (AGE) characterized up to now are produced by the combination of glycation and oxidation reactions, also called glycoxidation (Baynes, 1991; Fu et al., 1994). As a matter of fact, the consequences of posttranslational alterations of proteins due to oxidative processes have been less intensively investigated, being generally of more recent knowledge. For instance, proteins may be dramatically modified by interactions with lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal (Slatter et al., 2000). These products, as well as those derived from the simultaneous glycoxidation processes (ketones, aldehydes), can actively react together, or bind to protein amino groups

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MDA, malondialdehyde; MDA/BSA, malondialdehyde-modified bovine serum albumin; MDA/FCS, malondialdehyde-modified fetal calf serum; TUNEL, TdT-mediated dUTP nick-end labeling.

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and generate adducts and cross-links that significantly alter protein structure (Esterbauer et al., 1991). This has been well demonstrated in the case of MDA binding to type I collagen (Slatter et al., 1999).

Such processes are increased in various diseases, and the involvement of MDA binding to collagen was hypothesized in the degenerative complications of diabetes mellitus (Slatter et al., 1999, 2000). MDA concentration is elevated in the plasma of diabetic patients (Gallou et al., 1993), and MDA binding capacity to glycated proteins is higher than to non-glycated ones (Mooradian et al., 1996). Moreover, lipid peroxidation has also been shown to be directly promoted by glycated proteins (Hicks et al., 1988) or by oxygen free radicals generated by glycated proteins (Gillery et al., 1988; Ortwerth et al., 1998; Tavazzi et al., 2000). Thus, it was of interest to specifically study the effects of MDA binding to collagen on its interactions with fibroblasts, as an in vitro model of increased posttranslational modifications found in vivo. We have previously demonstrated that collagen matrix deeply influenced fibroblast functions, particularly by controlling protein synthesis (Gillery et al., 1995, 1996), and that these interactions were altered by nonenzymatic glycation (Rittié et al., 1999). Previous studies of literature have described the effects of free MDA on cell behavior, but nothing is known about the effect of MDA-modified proteins, which have increased concentration and half-life in living tissues when compared to free MDA (Sui and Draper, 1992), on cell metabolism.

In this study, we investigated the effects of MDA binding to both soluble and insoluble extracellular proteins on the regulation of fibroblast functions, and showed that this posttranslational modification strongly altered the ability of proteins to ensure a correct homeostasis of cultured cells.

MATERIALS AND METHODS

Reagents

Chemicals, peptides, and proteins (PDGF-BB, bovine serum albumin [BSA]), and TMP (1,1,3,3-tetramethoxypropane or malonaldehyde bis[dimethylacetal]) were purchased from Sigma (St. Louis, MO). Reagents for cell cultures and DNase I were from Life Technologies (Cergy-Pontoise, France). Cultures materials (culture flasks, Petri dishes, LabTek™) were from Merck-Eurolab (Strasbourg, France). FITC-Annexin V and the Homogeneous Caspases Assay-Fluorimetric kit were provided by Roche Diagnostics (Meylan, France), and the Apoptosis Detection System-Fluorescein was from Promega (Charbonnieres, France). The anti-Ki-67 antibodies were from Dakopatts (Glostrup, Denmark). Acid-soluble type I collagen was prepared from Sprague-Dawley rats tails as described elsewhere (Piez et al., 1963), sterilized with 70% (v/v) ethanol, and dissolved in 0.018 M acetic acid.

MDA-modified proteins

MDA solutions were freshly prepared by acid hydrolysis of TMP in a 0.15 M NaCl solution containing 0.18 M HCl. The various concentrations of MDA were expressed as final concentrations, considering that 1 mole of TMP produced 1 mole of MDA. After a 15 min incubation at room temperature, the pH of the solution was adjusted to 7.4 with NaOH and MDA solutions were, if necessary, sterilized by filtration.

Forty-five milliliters of fetal calf serum (FCS) or 40 g/L BSA solution in 0.15 M NaCl were incubated at 37°C for 3 h with 4.5 ml of MDA solution at concentrations of 0, 20, or 100 mM. The reaction was stopped by dialysis against 0.15 M NaCl to completely eliminate free MDA, and protein concentration adjusted at 30 g/L with 0.15 M NaCl. We used the terms MDA/FCS and MDA/BSA to qualify FCS or BSA incubated with MDA, and 20-MDA/FCS (or 20-MDA/BSA) and 100-MDA/FCS (or 100-MDA/BSA) to describe FCS (or BSA) incubated with MDA at a final concentration of 20 and 100 mM, respectively. The degree of protein modification was evaluated using thiobarbituric acid assay (Carbonneau et al., 1991). 100-MDA/FCS and 100-MDA/BSA contained 4.8 and 6.5 mol MDA incorporated per mol of total proteins (average molecular mass considered 50,000) and BSA, respectively. MDA/FCS or MDA/BSA were frozen at −20°C in aliquots and thawed just before use.

Cell cultures

Fibroblast strains were established from explants of human adult skin biopsies obtained from healthy volunteers with informed consent, cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS according to standard procedures (Gillery et al., 1992), and used between subcultures 3 and 9.

Collagen lattice cultures

Collagen lattice cultures were performed as previously described (Gillery et al., 1986) or with slight modifications for cultures using MDA-modified collagen. A 0.5 ml of type I collagen solution (4 mg/ml) were mixed on ice with 1.16 ml of 0.15 M phosphate buffer, pH 7.4, and 0.09 ml of 0.1 M NaOH, transferred into a 35 mm-diameter bacterial Petri dish and incubated overnight at 37°C to allow collagen fibrillation. Collagen lattices were then incubated with 1.25 ml of a 100 mM MDA solution for 3 h at 37°C, and extensively rinsed with 0.15 M NaCl until free MDA was undetectable in the medium (Carbonneau et al., 1991). In these conditions, the extent of collagen modification corresponded to 3.0 mol MDA incorporated per mol of collagen. Before cell seeding, collagen lattices were equilibrated in DMEM for 24 h at 37°C. Two hundred and fifty microliters of fibroblast suspension (10^5 cells per lattice) in DMEM containing 10% (v/v) FCS were deposited onto collagen lattices and incubated for 2 h at 37°C to allow cell attachment. One milliliter of culture medium was then added and lattices were detached from the walls of the dishes. Their diameter was measured every day using a graduated rule, the dishes being placed on a black background (Gillery et al., 1986).

Cell adhesion assay

96-well-culture plates were coated with type I collagen solution (25 µg/well) and rinsed three times with 0.15 M NaCl solution. Then, the collagen coat was incubated with 0, 5, 50, and 100 mM MDA solutions for 3 h at 37°C and rinsed three times with 0.15 M NaCl solution. Fibroblasts (5×10^4 cells) were plated and incubated for 0–90 min. The numbers of adherent cells were determined by nuclei staining with crystal violet

and results expressed in terms of absorbance at 560 nm (Gillies et al., 1986).

Proliferation, viability, and cell cycle assays

Fibroblasts (2×10^4 cells) were seeded into 24-well-culture plates in DMEM containing 10% (v/v) untreated or MDA/FCS. Every day, cells were counted with a hematocytometer and cell viability determined by Trypan Blue exclusion test.

Fibroblast proliferation status was determined by flow cytometry as described previously (Gorisse et al., 1999). Briefly, cells were trypsinized and centrifuged at 400*g* for 10 min, then fixed and permeabilized with 95% acetone. Cells were incubated with a monoclonal anti-Ki-67 antibody for 1 h and, after centrifugation and washing, with a Fluorescein Isothiocyanate (FITC)-labeled $F(ab')_2$ goat antibody for 30 min. After centrifugation and washing, cells were incubated in PBS containing 50 µg/ml propidium iodide and 1 mg/ml RNase for 1 h, in the dark at 4°C. Samples were analyzed by fluorescence-activated cell sorter (FACS) on a EPICS® MCL-XL (Coultronics, Beckman Coulter, Villepinte, France). The percentages of cells in G_0/G_1, S and G_2/M phases of the cell cycle were calculated from histograms.

Study of apoptosis features

FITC-annexin V/PI double staining for FACS analysis. Cells cultured in DMEM containing 10% (v/v) 0-, 20-, or 100-MDA/FCS for 2, 4, or 7 days were detached with 0.05% (w/v) trypsin-1 mM EDTA in PBS for 2 min at 37°C, washed twice with PBS and suspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 2% (w/v) FITC-Annexin V and 1 µg/ml PI. Cell suspensions (10^6 cells/ml) were then incubated for 10 min in the dark at room temperature and 400 µl of binding buffer were added to 200 µl cell suspension just before FACS analysis, as described above. A necrosis control was performed by incubating cells for 1 min with 50% (v/v) DMSO before analysis.

Determination of caspase activity. Homogeneous caspase activity (activated caspases 2, 3, and 6–10) was quantified by fluorimetry following the manufacturer's recommendations. Briefly, fibroblasts were cultured in 96-well plates in DMEM containing untreated or MDA-treated FCS for 2 or 4 days. Caspase substrate, pre-diluted in lysis buffer, was then incubated for 2 h at 37°C. The cleavage of the substrate was then evaluated by measuring the developed fluorochrome that is proportional to the concentration of activated caspases, using a Perkin Elmer HTS 7000 Plus multiplate reader spectrofluorometer (Lyon, France), with excitation and emission wavelengths set at 485 and 535 nm, respectively. A calibration curve was obtained using free R110 fluorochrome. Caspase-8 and caspase-9 activities were evaluated in separate series, on fibroblasts treated for 4 days with MDA-FCS, using specific colorimetric assay kits (Chemicon International, Inc.; Temecula, CA, ref. APT129 and APT139, respectively). The experiments were performed according to manufacturer's instructions. Assay specificity was assessed using specific caspase-8 (Ac-IETD-CHO) and caspase-9 (Ac-LEHD-CHO) inhibitors contained in the kits. The results were expressed as a ratio of $A_{405\ nm}$ to total cytoplasmic proteins (Bradford, 1976).

TUNEL (TdT-mediated dUTP Nick-End Labeling) assay for DNA fragmentation. Fibroblasts (10^4 cells) were plated into each well of a 4-chambers slides (LabTek®) in 500 µl DMEM containing 10% (v/v) 0-, 20-, or 100-MDA/FCS and incubated for 2, 4, or 7 days at 37°C. After incubation, cells were processed for TUNEL assay as recommended by the manufacturer. The images were captured using a fluorescence microscope (Axioskop 20, Zeiss Strasbourg, France) (filters set for green (520 nm) and red (630 nm) fluorescence) and processed using a digital camera (LH 750-RC3, Lhesa Electronique) and a PhotoMat 2.0 program (Multivision Instruments). A positive control of DNA fragmentation was made by incubation of Triton X-100-permeabilized cells with 0.1 U DNase I for 10 min before the addition of labeled nucleotides and TdT mixture.

DNA ladder. DNA fragmentation was checked by agarose gel electrophoresis of DNA extracted from human fibroblasts, incubated with either untreated or MDA-treated FCS during 2, 4, or 7 days, according to the method described by Gong et al. (1994).

Statistical analysis

Results were expressed as means of four determinations ± standard error of the mean (SEM). Statistical significance of the differences was studied using the Student's *t*-test.

RESULTS

Effect of MDA-binding to collagen and serum proteins on collagen lattice contraction by fibroblasts

When fibroblasts were seeded onto a 100 mM MDA-treated collagen lattice, a complete inhibition of contraction was noticed (Fig. 1). A strong although incomplete inhibition also occurred when MDA-treated FCS was added to the culture medium of lattices made of untreated collagen. For example, when fibroblasts were cultured in the presence of 10% 100-MDA/FCS, the

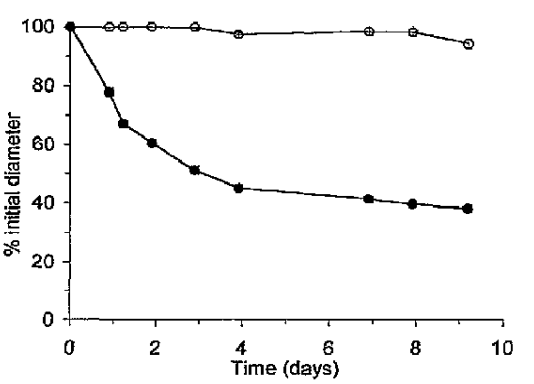

Fig. 1. Contraction of MDA-modified collagen lattices. Cells (10^5) were cultured in 100-MDA/collagen lattices (○) or in control collagen lattices (●), in DMEM containing 10% (v/v) FCS. Lattice diameter was measured every day. The results are the means of four determinations ± SEM.

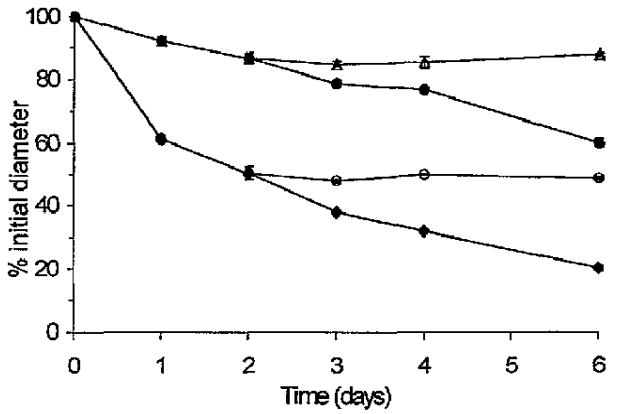

Fig. 2. Contraction of collagen lattices cultured with MDA/FCS. Cells (10^5) were cultured in a collagen lattice in DMEM containing 10% (v/v) control (◆) or 100-MDA/FCS (Δ). On Day 2, the culture medium was replaced in each series by DMEM containing 10% (v/v) control FCS (●) or 100-MDA/FCS (○). Lattice diameter was measured every day. The results are the means of four determinations ± SEM.

diameter of the lattice represented 87% of the initial diameter vs. 50% with untreated FCS ($P < 0.001$) on Day 2 (Fig. 2). At later times of incubation, fibroblasts cultured in the presence of 100-MDA/FCS did not contract the matrix any more. The inhibition of contraction was partially reversible. The replacement of 100-MDA/FCS-containing-incubation medium by fresh medium containing 10% control FCS on Day 2 restored contraction, with a kinetic similar to that observed in control lattices, reaching 60% of the initial diameter after 6 days vs. 88% in the 100-MDA/FCS series ($P < 0.001$). Likely, contraction of control lattices was stopped when incubation medium was replaced on Day 2 by fresh medium containing 10% 100-MDA/FCS: on Day 6, the maximal contraction observed was 48% of the initial diameter, vs. 20% in the control series ($P < 0.001$).

Effect of MDA-binding to collagen and serum proteins on fibroblast adhesion and multiplication in bidimensional cultures

In order to determine if the inhibition of contraction depended on the increase of mechanical resistance of the matrix or on specific effects of MDA adducts on cellular functions, we performed bidimensional cultures using MDA-modified proteins, under various conditions.

When fibroblasts were plated onto MDA/collagen coated plates, no significant difference of fibroblast adhesion, as measured by nuclei staining with crystal violet at 560 nm, was noticed when compared to adhesion on untreated collagen, whatever the level of MDA-induced modification (Fig. 3A). As well, fibroblast growth on MDA/collagen coats was not significantly altered (Fig. 3B). In all experiments, cell viability was higher than 93% (data not shown).

The influence of MDA modified-extracellular soluble proteins on fibroblast metabolism was then studied. The presence of 10% (v/v) MDA-treated FCS induced a dose-dependent inhibition of cell growth, reaching a total inhibition in 100-MDA/FCS series (Fig. 4A). The addition of PDGF-BB (50 ng/ml) did not reverse the inhibitory effects of MDA/FCS (data not shown). When culture medium contained 1 g/L MDA/BSA in addition to 10% (v/v) untreated FCS, a significant inhibition was observed after a 4 days incubation period (Fig. 4B).

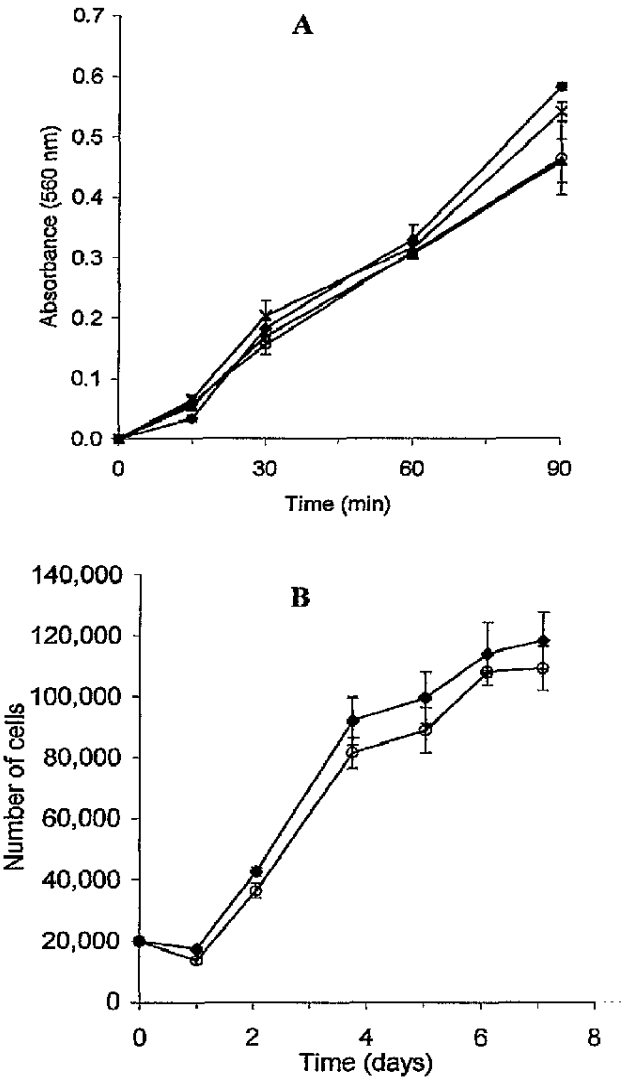

Fig. 3. Adhesion and proliferation of dermal fibroblasts on MDA-modified collagen. A: Adhesion: 5×10^4 cells were seeded onto unmodified collagen (◆) or on 5- (X), 50- (Δ), or 100- (○) MDA/collagen. The number of adherent cells was determined by crystal violet staining and expressed as $A_{560\ nm}$. B: Proliferation: 5×10^5 cells were seeded onto 100-MDA/collagen-coated plates (◆) and cultured in DMEM containing 10% (v/v) FCS. Unmodified collagen was used as control (○). The results are the means of four determinations ± SEM. No significant differences were found.

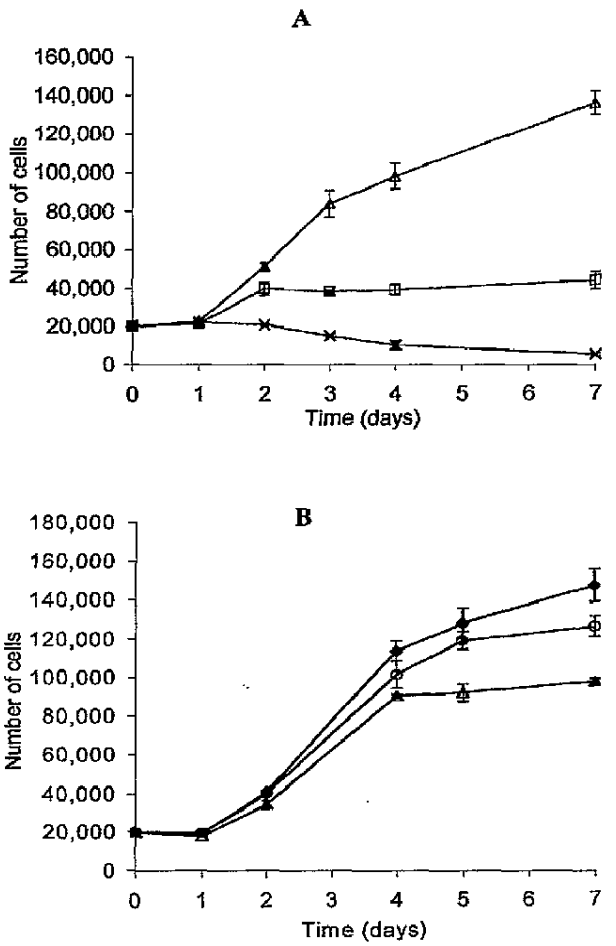

Fig. 4. Proliferation of dermal fibroblasts in the presence of MDA/FCS (A) or MDA/BSA (B). Fibroblasts (2×10^4) were seeded onto 24-well plates and cultured in: (A) DMEM containing 10% (v/v) 0- (Δ), 20- (■) or 100- (X) MDA/FCS (B) 10% (v/v) FCS and 1 g/L 0- (◆), 20- (○), or 100- (Δ) MDA/BSA. The results are the means of four determinations ± SEM.

The culture of fibroblasts in the presence of 10% (v/v) 100-MDA/FCS induced a significant alteration of fibroblast morphology. Whereas cells appeared elongated, well expanded, and spindle-shaped in control series, they appeared more swelled in MDA/FCS series, with an increased number of cellular expansions (data not shown).

Effect of MDA-modified proteins on apoptosis features

Morphological changes of cell shape being consistent with an induction of apoptosis, we studied early characteristic events of this process, such as the loss of phospholipid asymmetry, with exposure of phosphatidylserine on the outer leaflet. Flow cytometric analyses of cell permeability and membrane phospholipids distribution were performed using a double staining with annexin V, that preferentially binds to negatively charged phospholipids such as phosphatidylserine, and PI to evaluate the integrity of plasma membrane (Koopman et al., 1994). On Day 2 of culture, the number of cells stained with annexin V was largely increased in the presence of 100-MDA/FCS but not 20-MDA/FCS (Fig. 5). After 7 days of culture, the binding of annexin V was still very intense in 100-MDA/FCS cultured cells, and was increased in 20-MDA/FCS series when compared to control series. Meanwhile, neither changes in PI profile nor cell necrosis features were concomitantly noticed. Thus, MDA/FCS induced a time and dose-dependent loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine at the external cell surface.

Another early event of apoptosis is the activation of caspases (cysteinyl-aspartic-acid-proteases), which cleave several cellular components related to DNA repair and regulation. In order to check if MDA-modified proteins were able to activate these proteases, we used a fluorometric assay to quantify global caspases activity. A dose-dependent enhancement of caspases activity was induced by MDA/FCS and MDA/BSA (Fig. 6). For example, after 4 days of culture, 100-MDA/FCS increased caspases activity by 115 % vs. control series ($P < 0.05$), whereas 20-MDA/FCS exerted a slighter effect (+ 13%, NS). In the same way, the presence of 100-MDA/BSA (1 g/L) in culture medium induced a 91% increase of global caspases activity vs. control series ($P < 0.001$).

In addition, we investigated the potentially involved pathways by evaluation of caspase-8 and caspase-9 activities. The treatment of fibroblasts with MDA-modified FCS triggered a large dose-dependent increase in caspase-8 activity (Fig. 7A), whereas caspase-9 activity only slightly increased under the same experimental conditions (Fig. 7B).

The most common late event of apoptosis is fragmentation of DNA into ≈ 180 bp fragments. In order to detect internucleosomal cleavage of DNA, TUNEL assays were performed under the same culture conditions as above. There was no significant change in labeling after 4 days of culture and no DNA laddering was evidenced by agarose gel electrophoresis.

Effect of MDA-modified proteins on cell cycle arrest

As MDA/FCS and MDA/BSA significantly altered cell growth, we studied the proliferative activity of fibroblasts. The fraction of proliferative cells (Ki-67 positive cells) was determined by cell labeling with a mAB to Ki-67, identifying a nuclear protein complex expressed through all non-G_0 phases of the cycle. Ki-67 expression was analyzed by flow cytometry (Fig. 8). In control series, the percentage of Ki-67 positive cells showed a peak at 24 h, that did not exceed 50% of the whole population, and returned at the basal level (< 10%) at 48 h. The presence of 20-MDA/FCS or 100-MDA/BSA significantly decreased the number of Ki-67 positive cells at 24 h (28 and 14% of proliferative cells, respectively); 100-MDA/FCS also induced a decrease of the population of Ki-67 positive cells: the peak of proliferation, reaching 26% of the whole population, was, in addition, earlier

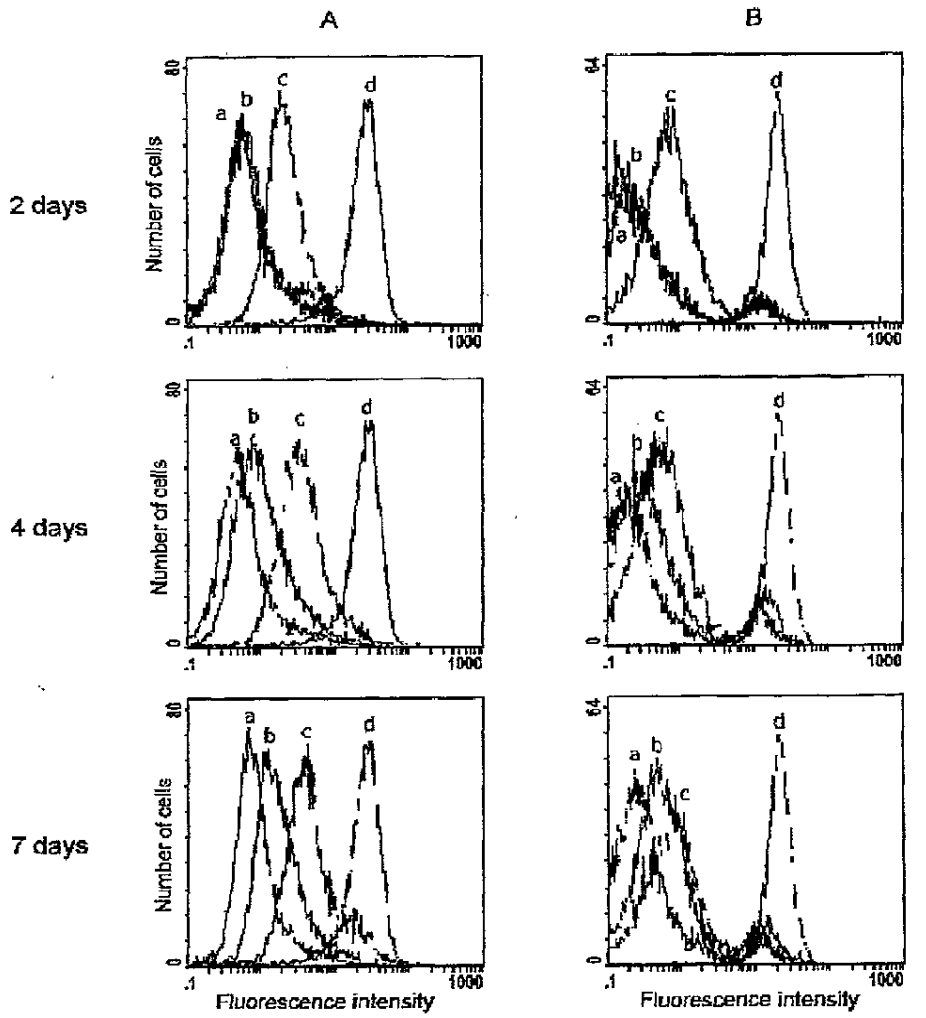

Fig. 5. Annexin V-FITC FACS analysis of membrane phospholipids exposure of dermal fibroblasts cultured with MDA/FCS. Cells were cultured in the presence of 10% (v/v) untreated (a), 20- (b), or 100- (c) MDA/FCS for 2, 4, or 7 days. Samples were prepared and analyzed by FACS and a control of cell necrosis was performed by cell incubation with DMSO (d). A double staining with annexin V (A) and PI (B) was performed in each experiment to evaluate the integrity of the plasma membrane.

than that of other series (12 h incubation vs. 24 h) and showed a slower decay up to 48 h.

The percentages of cells in G_0/G_1, S and G_2/M phases of the cell cycle at various incubation times were calculated from flow histograms. In control series, more than 70% of cells were in G_0/G_1 phases, and the number of cells in S and G_2/M phases largely increased at 24 h with a concomitant decrease in the number of cells in G_0/G_1 phases.

The pattern observed in the presence of 20-MDA/FCS was similar to that of control series. By contrast, the number of cells in G_2/M phases on Day 2 did not increase in the presence of 100-MDA/BSA. A 100-MDA/FCS treatment of fibroblasts induced significant changes in the distribution of cells: a regular decrease of the number of cells in S phase was evidenced. No simultaneous change in the percentage of fibroblasts in G_0/G_1 phases was noticed, whereas the number of cells in G_2/M phases increased (Fig. 9).

DISCUSSION

MDA is a highly toxic by-product generated in vivo by different biochemical pathways, among which prostaglandin synthesis. Another major source is lipid peroxidation, which intensity depends on oxygen-derived free radical production (Sui and Draper, 1992; Spiteller, 1998; Slatter et al., 2000). MDA exerts extremely deleterious effects on cells and tissues, by inducing damages to DNA or cellular proteins (Leuratti et al., 1998; Refsgaard et al., 2000). Several in vitro studies have

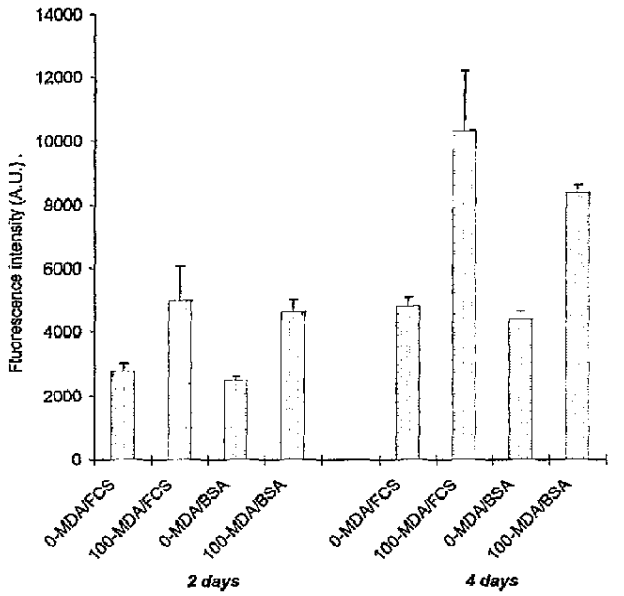

Fig. 6. Effect of MDA-treated proteins on global caspases activity in fibroblasts. Fibroblasts (10^5) were cultured in DMEM containing 10% (v/v) 0- or 100-MDA/FCS, or 10% (v/v) FCS containing 1 g/L 0- or 100-MDA/BSA. Caspases activity was measured by fluorometric assay. Results are the means of four determinations ± SEM.

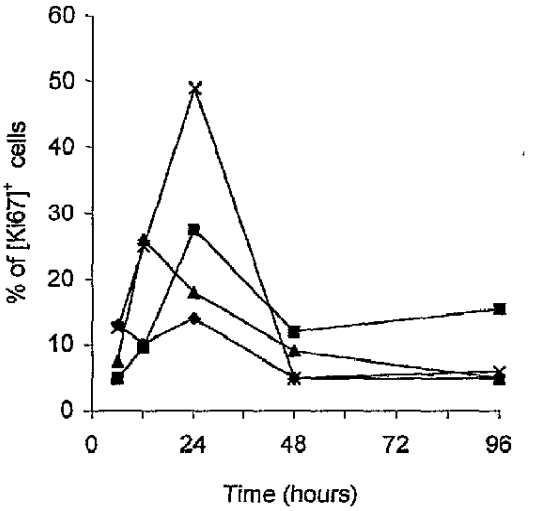

Fig. 8. Effect of MDA-treated FCS on cell proliferation evaluated by Ki-67 staining. Cells were cultured in DMEM containing 10% (v/v) 0- (X), 20- (■) or 100- (▲) MDA/FCS or in DMEM containing 10% (v/v) FCS and 1 g/L 100-MDA/BSA (◆). After treatment, cells were labeled with anti-Ki-67 antibody and the percentage of Ki-67 positive cells was evaluated by flow cytometry.

reported the effects of high concentrations of free MDA on various cell types (Jain, 1984; Ji et al., 1998). However, the biological half-life of free MDA is short in vivo, since MDA binds to proteins very quickly after its generation. This process occurs faster when proteins are already modified by nonenzymatic glycoxidation (Mooradian et al., 1996). Long-lived proteins such as type I collagen demonstrated major targets of MDA binding and accumulation, leading to the formation of crosslinks and to alterations of function (Slatter et al., 1998). Besides, preliminary unpublished results have suggested that MDA-collagen affected both the morphology and the expression of the cells (Slatter et al., 2000), as already demonstrated in the case of collagen modification by advanced glycation end products (AGE) (Paul and Bailey, 1999). In this context, we investigated the consequences of MDA-binding to collagen and other proteins on metabolic functions of fibroblasts, using tri- and bidimensional culture models. Our starting point was the culture in collagen lattices, which are considered valuable in vitro models of in vivo cell-matrix interactions (Gillery et al., 1986, 1989).

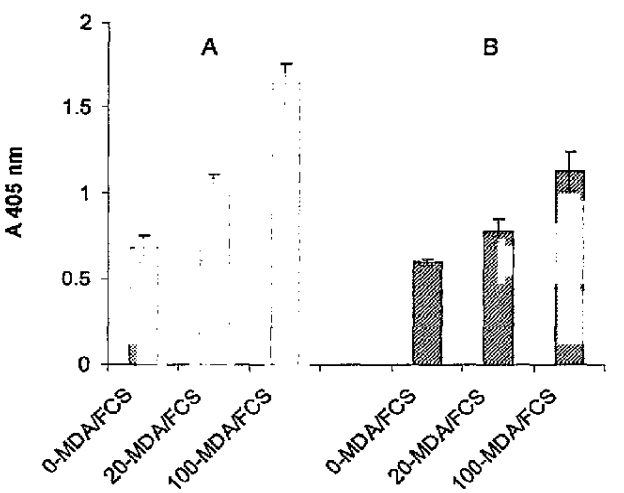

Fig. 7. Effect of MDA-treated FCS on caspase-8 (A) and caspase-9 (B) activities. Fibroblasts (5×10^5) were cultured in DMEM containing 10% (v/v) 0-, 20-, or 100-MDA/FCS for 4 days. Caspase-8 and caspase-9 activities were measured by colorimetric assays. Results are the means of three determinations ± SEM.

When seeded in MDA-treated collagen gels, fibroblasts are no more able to efficiently contract the matrix, which may be explained by several features. Various hypotheses could be ruled out, such as a decrease of viability or an impairment of fibroblasts adhesion to the modified substrate, as demonstrated by 2-D cultures on collagen coats. Accordingly, inhibition of matrix remodeling was not due to changes in secretion or activation of MMPs (data not shown), contrary to the case of glycated collagen lattices (Rittié et al., 1999). These data support the hypothesis that the inhibition of matrix contraction mainly derives from an increased resistance of the network induced by MDA-generated cross-links (Slatter et al., 2000), but do not rule out the possibility that MDA adducts directly alter interactions with cell membranes. In this regard, we showed that

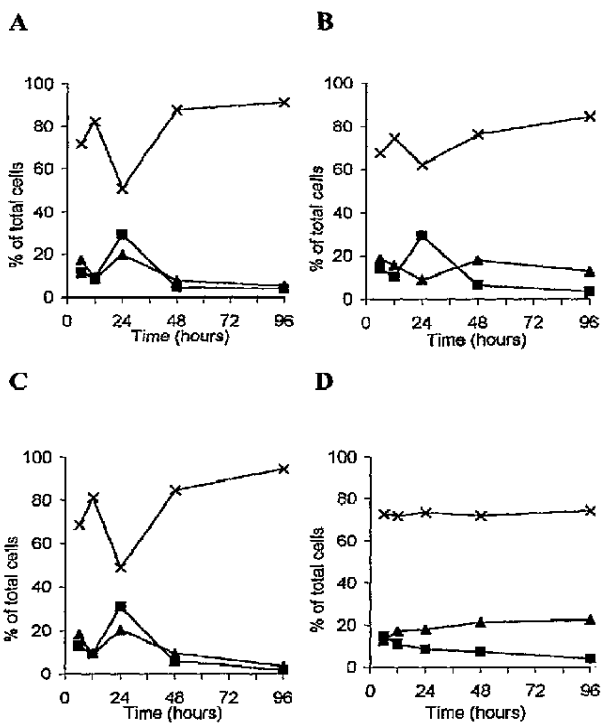

Fig. 9. Effect of MDA-treated FCS on cell cycle distribution. Cells were cultured in DMEM containing 10% (v/v) 0- (A), 20- (C), or 100- (D) MDA/FCS or in DMEM containing 10% (v/v) FCS and 1 g/L 100-MDA/BSA (B). After treatment, DNA was labeled with propidium iodide and the percentage of cells in G_0/G_1 (X), S (■), or G_2/M (▲) phases was determined by flow cytometry.

MDA-modified serum proteins also inhibited collagen lattice contraction, in a partially reversible manner. This suggested that the influence of MDA adducts by themselves is at least as important as the nature of the modified protein. Furthermore, MDA modified-soluble proteins induced additional deleterious effects on fibroblasts, by triggering a dramatic decrease of mitotic activities and the induction of apoptotic processes. A complete inhibition of cell growth was noticed, together with major alterations of cell morphology, resembling replicative senescence. The number of cells remaining in culture progressively decreased in a dose-dependent manner. Similar effects were triggered either by MDA-modified FCS or BSA, for comparable amounts of MDA adducts, suggesting that interactions of MDA adducts with cell membranes occurred independently from protein specificity. Another hypothesis was that MDA treatment might inactivate FCS growth factors necessary to cell multiplication. This hypothesis was not confirmed, at least in the case of PDGF-BB, which could not restore cell growth of fibroblasts exposed to MDA/FCS. However, other factors are probably involved, since the kinetics of MDA/BSA and MDA/FCS effects were different, even in the presence of comparable amounts of MDA adducts.

We could not demonstrate that MDA adducts induced a complete apoptosis process: early steps were noticed, such as increase of annexin V affinity binding, reflecting the flip-flop of phosphatidylserine in the plasma membrane and its outer leaflet exposure (Koopman et al., 1994), as well as an induction of the caspases activities. Assays of specific caspases activities showed a preferential effect of MDA-modified FCS on caspase-8 activity, suggesting the primary involvement of the receptor pathway. Nevertheless, the mitochondrial pathway might also be implicated during prolonged incubations, as suggested by the slight increase in caspase-9 activity elicited by MDA-modified FCS. The involvement of the mitochondrial pathway could be induced by active caspase-8 (Hengartner, 2000). However, no further DNA fragmentation was noticed. It seems however that loss of phospholipid asymmetry and PS exposure can be divorced from other changes associated with apoptosis (Fadok et al., 2001), and that apoptosis induced by lipid mediators may show unusual characteristics (Haynes et al., 2001). Besides, many reports underline that apoptosis detection depends on the characteristics of the method used (Otsuki, 2000), which conditions the interpretation of data. In our study, early apoptotic features are concomitant with major alterations of cell proliferating activity and cell cycle progressing. At 48 h, about 90% of cells were quiescent in control cultures. The treatment of cells with MDA/FCS induced an inhibition of the proliferating activity, and changes occurred earlier than in control series. The Ki-67 positive cell fraction was decreased about fivefold by a 24 h 100-MDA/BSA treatment. The simultaneous occurrence of decreased mitotic activity and increased number of cells in the G_2/M phases suggests an arrest of cell cycle. Such major alterations of cell metabolism in adherent, proliferative cells, might explain the progressive decrease of cells remaining in culture.

Previous studies have reported the induction of apoptosis by free MDA, but only in tumor cells, *i.e.*, colorectal and lung carcinoma cells (Ji et al., 1998). We show here for the first time that MDA bound to proteins can induce a similar effect in normal cells. The mechanism of this alteration is still speculative. The initial event could be a disorganization of plasma membrane, or interactions with other intracellular proteins, as demonstrated in the case of free MDA (Jain, 1984; Kikugawa et al., 1984). This hypothesis does not rule out the possibility of triggering other mechanisms besides apoptosis, such as specific intracellular signaling, as demonstrated for 4-hydroxynonenal, another by-product of lipid peroxidation (Dianzani, 1998; Ruef et al., 2001).

Collectively, our data show that stable MDA adducts are of potential toxicity for normal cells, as well as free MDA. The long biological half-life of MDA-modified proteins is a pejorative factor that strongly suggests their involvement in degenerative complications of aging and of various diseases. The most popular example is diabetes mellitus, where MDA production (Baynes, 1991, 2000; Gallou et al., 1993; Lyons and Jenkins, 1997; Vlassara, 1997; Bailey et al., 1998; Bailey and Paul, 1999; Slatter et al., 2000), serum concentration (Carbonneau et al., 1991) and tissue content (Shak et al., 1994) are increased concomitantly with the intensity of glycoxydation. It is well accepted that posttranslationally

modified proteins alter metabolic functions of mesenchymal (Rittié et al., 1999) as well as of inflammatory cells (Scatena et al., 1998; Monboisse et al., 2000). Moreover, MDA-altered proteins have been directly involved in the constitution of atheroma lesions (Haberland et al., 1988; Hill et al., 1998). As well, circulating proteins containing MDA adducts are involved in this process. This has been well demonstrated for oxidized low density lipoproteins (LDL), which are taken up by macrophage scavenger receptors, leading to foam cell formation (Berliner and Heinecke, 1996). In addition, oxidized LDL induce deleterious effects on various cell types, particularly endothelial cells (Penn and Chisolm, 1994), and significantly alter the expression of adhesion molecules (Lin et al., 1996; Smalley et al., 1996). Moreover, antibodies against oxidized LDL are predictive of the progression of atherosclerosis (Holvoet et al., 2001). Our results clearly confirm that MDA-binding to structural or circulating proteins strongly alters their ability to modulate cell metabolism and ensure tissue homeostasis, and demonstrate the intrinsic toxicity of protein-bound MDA, in culture models close to physiological conditions and clinically more relevant than previous studied performed with high concentrations of free MDA.

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